Journal of Medical Virology 49:199–204 (1996)

Glial and Muscle Embryonal Carcinoma Cell-Specific Independent Regulation of Expression of Human JC Virus Early Promoter by Cyclic AMP Response Elements and Adjacent Nuclear Factor 1 Binding Sites

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The human polyoma JC virus (JCV) is a glial cell-specific virus and is the etiological agent for the terminal AIDS-associated brain disease, progressive multifocal leukoencephalopathy (PML). JCV contains several binding sites for transcriptional factors that are important for activity in glial cells, including cyclic AMP (cAMP) response elements (CREs) which are four nucleotides from nuclear factor 1 (NF1) sites within the two 98 bp repeat regions. We studied the combined role of cAMP and NF1 in regulating the expression of the JCV early promoter-enhancer (JCV_E) in differentiating glial and muscle P19 embryonal carcinoma cells. JCV_E expression remained several-fold higher in the presence of cAMP in glial cells, irrespective of whether the relatively strong activity of JCV_E was greatly reduced by NF1 site mutations. In contrast, cAMP had no effect in muscle cells, independent of whether the modest activity of JCV_E was two-fold higher due to NF1 site mutations. The in vivo effects were confirmed with in vitro transcription assays using glial cell extracts, competitors of CRE, and the NF1 site, and single repeat JCV_E region with mutations in the NF1 II/III binding sites as templates. The in vitro results also indicated that the effects were due to the CREs of JCV, rather than to the indirect effects of cAMP. Overall, the results indicated that NF1 and cAMP have independent, different, tissue-specific, and direct effects in the regulation of JCV_E. These effects may contribute the neurotropic PML-inducing pattern of expression of JCV_E.

KEY WORDS: transcription regulation, neurotropic, glial cell specificity, binding sites

INTRODUCTION

The human JC polyomavirus (JCV) was originally identified as the etiological agent for the fatal demyelinating brain disorder called progressive multifocal leukoencephalopathy (PML) and was found to be highly glial cell-specific for growth [for review, see Frisque and White, 1992]. The regulatory region for the neurotropic gene expression of JCV consists mainly of two 98 bp repeats, which have binding sites for several transcription factors [Major et al., 1992; Ranganathan and Khalili, 1993; reviewed by Raj and Khalili, 1995]. Using site-directed mutagenesis, independent roles in the induction of glial cell-specific viral expression were suggested for the JCV cyclic AMP (cAMP) response elements (CREs) (Kumar et al. 1996) and the closely adjacent nuclear factor 1 (NF1) binding site sequences in the 98 bp repeat region [Kumar et al., 1993]. In addition to CREs and NF1 sites, the binding sites for several other factors, including those for Tst1, the Sac-1 motif factor, Sp1, and the *Jun*-related factor, have been shown to play roles in the transcriptional regulation of JCV in glial cells [Wegner et al., 1993; Tamura et al., 1990; Henson, 1994; Amemiya et al., 1992]. However, how the combination of factors function in controlling JCV expression in glial cells has remained unclear.

The function of cAMP is notably distinct from the function of NF1. cAMP mediates the action of peptide hormone receptors at the cell surface. After the receptor–hormone binding, the signal transduction pathways re-

Mary M. Pater passed away on November 2, 1994 and this paper is dedicated in her fond memory.

Accepted for publication February 16, 1996.

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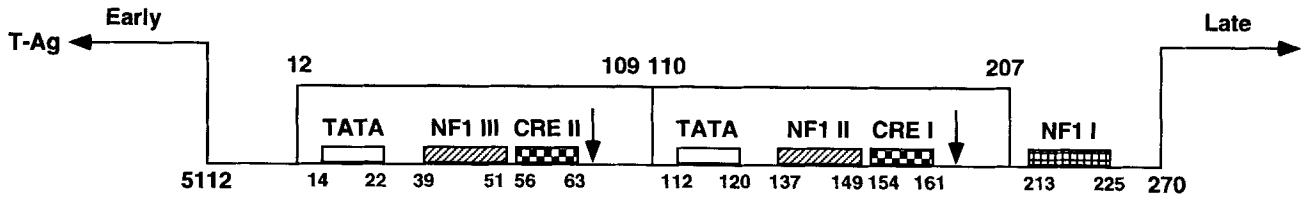

Fig. 1. Diagram of JCV regulatory region sequences relevant to current study. Shown are: large boxes, 98 bp repeats; small labelled boxes, binding sites for various transcription factors; horizontal arrows and Early and Late, early and late directions of transcription; vertical arrows, boundaries of the sequences inserted into the pRII and pmRII single 98 bp repeat JCV expression vectors [Kumar et al., 1993]; numbers, JCV nt; lower line, sequences inserted into the JCV_E expression vectors containing JCV_E without (WT) or with (I, II, II.III, and I.II.III) the NF1 mutations [Kumar et al., 1993]. Roman numerals of NF1 motifs indicate the mutated sites.

lay the signal to second messengers, such as cAMP [Roesler et al., 1988]. Consequently, CRE binding proteins (CREBs) are activated by phosphokinases. Interestingly, the brain-specific CRE-BP1/ATF-2 type of CREB in the human brain forms heteromers with c-*jun* at their binding sites [Ivashkiv et al., 1990]. Generally, CREB interacts with several tissue-specific factors. For example, CREB was shown to interact with other tissue-specific factors, such as the protein that binds to the placental tissue-specific element located adjacent to CREs in the alpha chorionic gonadotropin promoter [Delegeane et al., 1987]. The two latter studies indicated that the proximity of the adjacent sites is important for the interaction of CREB with the other factors, resulting in efficient cell-specific transcriptional activity.

The binding sites of several genes that are specific to brain and other cell types have closely proximal sites that frequently include the motif for the NF1 transcription factor. Often other factors binding to the closely adjacent sites cooperate in regulating cell-specific gene expression [Amemiya et al., 1992; Jackson et al., 1993; Mink et al., 1992]. Since neurotropic JCV also contains two copies of NF1 binding site four nucleotides from the CRE, we examined the possible cooperation between NF1 and cAMP in the specific expression of JCV_E in glial and muscle P19 cells. Our results showed the following: 1) The effects of cAMP and NF1 in the expression of JCV_E were independent and different. 2) The regulation by both was highly cell-specific. 3) The function of cAMP for the JCV CRE was direct.

MATERIALS AND METHODS

Plasmids

The JCV_E reporter gene transient expression constructs, $pJCV_Ecat$(WT) and the same plasmid containing the NF1 site I, II, II.III, I.II.III, RII, and mRII mutations, were described previously [Kumar et al., 1993] and are described in Figure 1.

Cell Culture, DNA Transfection and Expression Assays

P19 embryonal carcinoma undifferentiated cells were treated with 1% dimethyl sulfoxide to differentiate into mostly skeletal and cardiac muscle cells and were differentiated into a mixture of neuronal, glial, and other cells with 300 nM retinoic acid (RA), as described earlier [Rudnicki and McBurney, 1987]. The majority of RA cells expressing transfected DNA are glial [Devireddy et al., 1996]. Undifferentiated, muscle, and RA glial P19 cells were transfected, as described previously [Nakshatri et al., 1990; Kumar et al., 1993]. For cAMP induction studies, P19 cells were cultured 24 hr after transfection in 100 μM forskolin (FSK) to increase the cAMP level, or 10 μM dibutyryl cAMP [Kumar et al., 1996]. Chloramphenicol acetyl transferase (CAT) transient transcriptional expression assays were corrected for the pSV0cat parental plasmid containing no JCV sequence, in calculating the fold effects of cAMP and NF1. Also, the CAT assays were normalized for the transfection and transcriptional efficiencies with β-galactosidase assays, as described previously [Gorman et al., 1982; Kumar et al., 1993].

In Vitro Transcription Assays

To obtain 550 bp run off transcripts, 1 μg *Nco*I-digested pRII and pmRII templates were used. The *Hpa*I-digested pRSV-β-galactosidase plasmid was used as an internal control for the recovery of transcript and transcriptional efficiency. The binding assays used 60 μg whole cell extracts and 5% sequencing gels. The 5′-GTCGACTGGCTGCCAGCCAA-3′ 20-mer NF1 II/III and the 5′-AGCATGAGCTCA-3′ 12-mer CRE oligonucleotides were used in the competition studies. Other conditions were described previously [Kumar et al., 1993].

RESULTS

In previous studies, we used site-directed mutagenesis of the control region of JCV to suggest a role for CREs [Kumar et al., 1996] and an independent role for the NF1 sites [Kumar et al., 1996] in the 98 bp repeat region of JCV in the P19 glial cell-specific expression of JCV. Here, the possibility of cell-specific cooperative interactions between the factors binding to CREs and the closely adjacent NF1 binding sites was examined. The expression vectors contained the whole JCV_E wild type (WT) and the mutations of the nonrepeat region NF1 site (I), both repeat region NF1 sites (II.III), or all 3 JCV NF1 sites (I.II.III). Also, the single repeat vector contained the wild type (RII) or a mutated (mRII) NF1 site (Fig. 1) [Kumar et al., 1993]. For undifferentiated and muscle P19 cells cultured in cAMP or FSK, no induction of expression by cAMP was detected for JCV_E, irrespective of the mutation of the NF1 sites (Fig. 2). No effect of

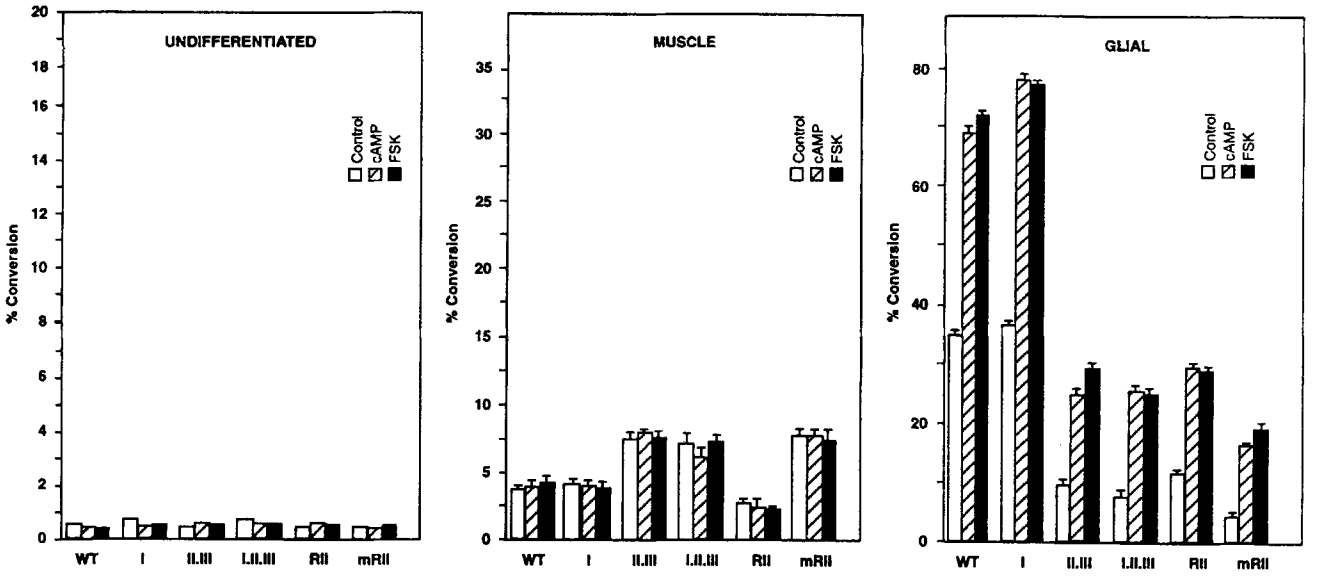

Fig. 2. Cell-specific effect of cAMP and NF1 on the expression from JCV_E sequences. The assays used wild type and mutated NF1 site in undifferentiated and muscle and glial differentiated P19 cells, as indicated. The percent conversion for CAT expression plasmids for the whole enhancer-promoter region containing wild type (WT) or mutated NF1 motifs are indicated, as described in Figure 1. Also, the plasmids described in Figure 1 and containing motifs in the 98 bp repeat region with wild type NF1 II (RII) and mutated NF1 II (mRII) are indicated. Also indicated, are control cells (Control); cAMP-treated cells (cAMP); and forskolin (FSK) cAMP-induced cells.

TABLE I. Effect of cAMP and Mutation of NF1 II Site on the Expression of JCV_E in Differentiating P19 Cells and Glial Cell Extracts

	P19 cells			Glial
Factor	Undifferentiated	Muscle	Glial	extracts
NF1	NS	−2.0-fold	+4.5-fold	+4-fold
cAMP	NS	NS	+2.5-fold	+2.4-fold
NF1 + cAMP	NS	−2.0-fold	+7.0-fold	+6.5-fold

*NF1, NF1 II site; NS, not significant; + and − signs, positive and negative responses, respectively.

mutated NF1 sites in undifferentiated cells and an increased activity for mutated sites in muscle cells was observed, as in previous results [Kumar et al., 1993] (Fig. 2, Table I). In contrast, all constructs gave increased activities for cAMP and for induction by FSK in the in vivo assays of glial P19 cells. The II.II and I.II.III mutations in NF1 sites had a marked negative effect on glial cell activity, as in previous results [Kumar et al., 1993], but had little effect on the JCV_E response to cAMP (Fig. 2). The fold inductions ranged from 2.1 for WT to 3.6 for I.II.III and from 2.7 for RII to 4.2 for mRII in glial cells (Fig. 2). The results indicated the cell specificity of both transcription factors. Moreover, they suggested that the regulation in glial cells by cAMP and NF1 of JCV_E is not affected by the juxtaposition of the two binding motifs; rather, the induction of JCV_E by cAMP and NF1 sites is independent (Table I).

To examine further the effects of cAMP and the NF1 motifs and to examine the direct effect of the JCV CRE on the functional expression of JCV_E, in vitro transcription was assayed with P19 glial cell extracts. Wild type RII 98 bp single repeat CAT expression plasmids gave 2.4-fold more densitometry-analyzed activity with FSK-treated glial cell extract than with untreated (RA) control cell extract (Fig. 3, RII). Competition with NF1 II/III or CRE oligonucleotides resulted in approximately 50% reductions of JCV_E activity. The mutation of NF1 II in mRII resulted in almost undetectable in vitro transcriptional activity with glial cell extracts, consistent with the expression in intact cells (Fig. 2, 3, mRII). However, increased activity was seen with extracts from cells induced for cAMP by FSK treatment (Fig. 3, mRII FSK). Competition with the CRE, but not NF1 II/III, oligonucleotide reduced the activity observed with FSK glial cell extracts to the almost undetectable level observed for RA glial control cells. The absence of competition by the NF1 II/III oligonucleotide (NF1) of binding to the mutated NF1 site (mRII) indicated the validity and confirmed the specificity of the partial competition by this oligonucleotide of binding to the intact NF1 site (RII). The complete competition by CRE for mRII indicated that the incomplete competition by CRE for RII was not a partial competition of the transcriptional function of the CRE. Overall, the in vitro transcription results confirmed the significant effects of both CRE and the NF1 site and suggested that the induction of JCV_E by cAMP

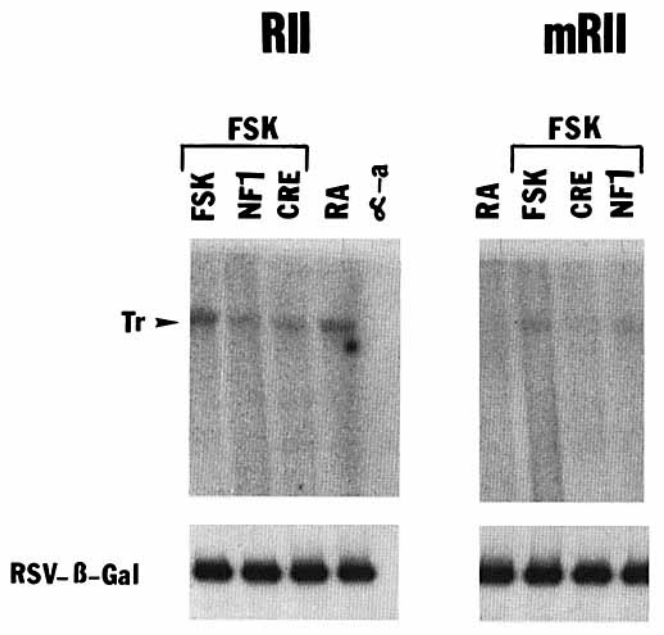

Fig. 3. Effect of NF1 and CRE motifs on expression of JCV_E in glial cell extracts. In vitro transcription assays are shown. Labels: RII and mRII, pRII and pRII CAT expression plasmid templates; upper FSK, extract from forskolin (FSK) cAMP-induced P19 cells; lower FSK, assays for FSK cells with no competitor; NF1 and CRE, competition with 250-fold excess JCV NF1 II/III and CRE oligonucleotides, respectively; RA, extract from control FSK-untreated cells; α-a, assay as for RA with 1 μg/ml α-amanitin RNA polymerase II inhibitor; Tr, CAT transcript; RSV-β-Gal, p-RSV-β-galactosidase transcript used as internal control for the recovery of CAT transcript and transcriptional efficiency.

is not dependent on NF1. Rather, the independent two-way competition by oligonucleotides was consistent with the results in P19 glial cells, showing the independent, unique and tissue-specific contribution of both motifs to the transcription function of JCV_E (Fig. 2, 3, Table I). Further, the in vitro results indicated that the effect was not the result of indirect cellular effects.

DISCUSSION

The transcription regulatory regions of several brain-specific genes contain NF1 binding sites and adjacent activator protein binding sites, such as CREs and the AP-1, ATF, and AP-2 sites [Amemiya et al., 1992]. In addition, the role of NF1 in cell-specific transcription has also been well documented [Gloss et al., 1989; Mink et al., 1992; Jackson et al., 1993]. In the context of the cell type-specific promoters and enhancers, NF1 proteins cooperate with other transcription factors binding to the sites located in close proximity to the NF1 binding sites. For example, HPV16 is another papovavirus, is strictly epitheliotropic, and contains an NF1 site that binds NF1 and was suggested to act cooperatively with an Oct-1 site that is two bp from the NF1 site [O'Connor and Bernard, 1995]. Other closely proximal HPV16 sites include a composite binding site, which contains an AP-1 non-receptor binding site and the glucocorticoid receptor binding site. Factors binding to the two sites were highly cooperative [Mittal et al., 1994].

Interestingly, NF1 binding sites with adjacent CREs are contained in the JCV regulatory region, which exhibits low to negligible expression in muscle cells and is strictly glial cell-specific for viral gene expression and replication [Nakshatri et al., 1990; Amemiya et al., 1992; Ault and Stoner, 1993; Kumar et al., 1996] (Fig. 1). In the present study, in vitro transcription assays showed the independent binding of CRE and the NF1 motif in glial cell extracts. The same pattern was observed for the regulation of JCV_E activity in glial cells. Hence, the CREB and NF1 that bind to the respective motifs appeared not to interact functionally with each other. Furthermore, NF1 played a minor role in gene expression for JCV_E in muscle cells. Cyclic AMP had no effect [Kumar et al., 1993; present study]. Thus, the JCV CRE is one of the brain-specific CREs [Ivashkiv et al., 1990].

EBNA2 is a transactivator of Epstein-Barr virus and VP16 of herpes simplex virus. Recently, EBNA2 was shown to interact with three of the RNA polymerase II general transcription factors (GTFs), TFIIB, TFIIE, and TFIIH, but not with the TATA box binding protein (TBP) of the TFIID multimeric complex [Tong et al., 1995a; Tong et al., 1995c] (Fig. 4) [for reviews, see Tjian and Maniatis, 1994; Maldonaldo and Reinberg, 1995]. However, EBNA2 also interacted with the TBP-associated factor (TAF), TAF40. Distinctively, the VP16 activator bound strongly to TBP and TFIIH [Tong et al., 1995a,b]. Interestingly, CREB was also shown to stimulate transcription by interacting with TFIIB and TFIID, but not TBP [Goodrich and Tjian, 1994; Xing et al., 1995]. Further, CREB interacted specifically with TAF110 [Ferreri et al., 1994]. On the other hand, the NF1-related C/EBPα was shown to interact with TBP, as well as with TFIIB [Nerlov and Ziff, 1995]. Thus, CREB and NF1 may independently up-regulate JCV_E via different components of the basal transcription apparatus. This hypothesis was supported by our previous observation that mutations of the CRE lead to a lack of response to cAMP [Kumar et al., 1996]. Moreover, the independent function of both factors was confirmed by our present results of expression assays in glial cells and cell extracts and in muscle cells. Consistently, differentiation responsive sequences acted independently for the tissue plasminogen activator gene, containing an adjacent CRE and NF1 site [Rickles et al., 1989].

A proposed mechanism for the glial cell-specific regulation of JCV_E via the CREB and NF1 transcription factors interacting with the basal transcription apparatus is diagrammed in Figure 4. The mechanism involves the direct regulation at the JCV_E TATA box through GTFs/TAFs/TBP/co-activators. For the role of co-activators, the binding of EBNA2 to the TFIIE GTF was via the p100 cellular co-activator [Tong et al., 1995c]. Hence, a coactivator mediating the JCV-specific and glial cell-specific effect is also suggested to permit the various interactions of the glial CREB and NF1 II/III (Fig. 4). The regulation in glial cells by the independent activation of JCV_E by CREB and NF1 up-regulates the expression of JCV large tumor antigen (T-Ag) and the mutation of the NF1 II/III site results in reduced T-Ag expression.

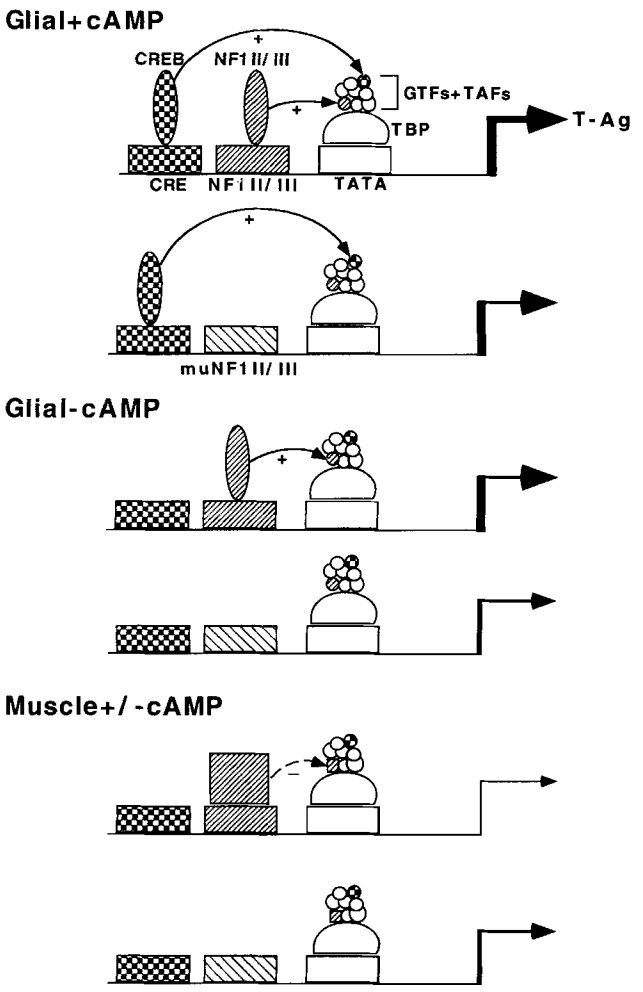

Fig. 4. Model for regulation of JCV_E in glial and muscle cells by CREB and NF1. The transcription factors above the line, the binding sites below the line and T-Ag are labelled the first time they are shown. The arrows from CREB and NF1 to GTFs and TAFs, and + and −, indicate positive and negative direct or co-activator-mediated effects on JCV_E expression. The muscle cell square NF1 and TAF represent possible muscle cell-specific factors, and the dashed line represents a possible muscle cell-specific co-activator. The sizes of the arrows on the right indicate the relative levels of JCV_E expression. See Figure 1 and the text for other details and abbreviations.

For JCV_E, expression of T-Ag is reduced a similar amount in the absence of cAMP and the additional mutation of the NF1 motif reduces expression a further similar amount.

The expression of JCV_E in muscle cells is altered dramatically, as for many other examples of cell-specific gene regulation (Fig. 4) [Roberts and Green, 1995]. Unique responses to NF1 mutations and cAMP were observed in muscle cells. The effect of mutating the NF1 site was negative in glial cells and positive in muscle cells, consistent with our previous report (Fig. 2) [Kumar et al., 1993]. Two explanations are suggested. First, the unique NF1s that bind the NF1 II/III site may regulate JCV_E negatively in nonglial cells and positively in glial cells, as reported previously (Fig. 4) [Tada et al., 1989; Kumar et al., 1993]. Second, a protein was found to bind and repress the Sp1 transcription factor [Murata et al., 1994]. Further support for such a repressor for the NF1 in muscle cells, is the down-regulation of JCV_E expression in cell hybrids [Beggs et al., 1988]. Therefore, the opposite effect of NF1 in muscle and glial cells could involve a muscle cell-specific repressor, NF1, NF1 II site, co-activator, or GTP/TAF/TBP. The same may apply for the differential response of the CREB interacting with the JCV CRE in glial and muscle cells.

The independent function of the factors binding to CRE and the NF1 binding site in the JCV regulatory region suggests that each factor stimulated JCV_E expression by unique mechanisms. Glial-specific expression of JCV_E may be tightly regulated and have a differential response to external signals and the physiological status of the cell, as follows. First, glial cells are known to be responsive to catecholamines through the cAMP second messenger [Stone and John, 1991]. Consequently, cAMP might induce the phsophorylated CREB to independently bind the CRE adjacent to the JCV_E NF1 II/III. Second, NF1 tightly regulates the control of gene expression by NF1's differential expression in various tissues, differential splicing and heterodimerization [Santoro et al., 1988; Rupp et al., 1990; Apt et al., 1994]. Both mechanisms could independently directly regulate the glial cell-specific expression of the CRE- and NF1 site-dependent JCV_E. An analogous mechanism has been proposed for the effect of cAMP on liver-specific gene expression [Roesler et al., 1993].

ACKNOWLEDGMENTS

The authors thank S. Atkins and J. Petten for typing the manuscript. This work was supported in part by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada (with funds from the Canadian Cancer Society).

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